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(57) Abstract

The invention relates to applications of haloperoxidases in substantially isolated form, obtainable from a large number of filamentous fungi, in paints and in a method for determining the halide concentration in a liquid. The haloperoxidases can be enzymes isolated from the fungi or produced recombinantly. In a preferred embodiment, the invention provides a paint with growth-inhibiting properties, for instance for use on the underwater part of ships. In addition, the invention provides a test kit for determining the halide concentration in a sample.

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Antifouling paint containing haloperoxidases and method to determine halide concentrations

The present invention relates to the use of haloperoxidases, particularly chloroperoxidases, in isolated form, whether or not obtained via recombinant DNA techniques, in a number of applications which relate to determining chloride in a solution and to different paints.

Haloperoxidases are enzymes which can convert halides into hypohalogenic acids. Haloperoxidases occur inter alia in a number of filamentous fungi and seaweeds. Now that such haloperoxidases have become available in isolated form, different applications have now been found to be possible.

A first aspect according to the invention is related to a problem which occurs with different painted surfaces, particularly with the parts of ships extending in the water, that is, the growth of unwanted organisms. Painted surfaces not exposed to water can also become for instance green on the outside through algal growth. The growth of algae and other organisms on and in painted surfaces reduces the life span of the coating of paint, inter alia because it will have to be repainted sooner from an aesthetic point of view. The drawback of fouling of ships is that the resistance of the ship in the water increases. This has a negative effect on the movement of the ship, which will cost more effort or will become slower. Ships are therefore treated with growth-inhibiting agents, so-called anti-foulings. Known anti-foulings are however environment-unfriendly and are also not always very effective.

It is therefore a first object of the invention to provide a new paint, with which the above stated drawbacks can be avoided.

According to the invention it has now been found that the addition of a haloperoxidase to paint can reduce or even prevent the growth of unwanted organisms on a painted surface by producing disinfecting hypohalogenic acids. To this end the invention provides paints which contain at least a suitable quantity of one haloperoxidase in addition

to the usual paint constituents and solvents. Understood here by "suitable quantity" is a quantity such that growth on a surface treated with the paint will be substantially prevented. Using his normal professional knowledge the skilled person will be capable of determining both the composition of the paint and the quantity of enzyme to be added.

In another embodiment of the invention the haloperoxidases can be used as preservative in paints. Particularly water-borne paints are subject to relatively rapid spoiling. Due to its disinfecting action the enzyme can ensure the storage life of such paints during storage and when they are being used.

The invention is based on the conclusion that in nature a number of vanadium bromoperoxidases are found on the surface of seaweeds. In the intact plant in seawater the vanadium bromoperoxidase is accessible to added substrate and is capable of forming HOBr after addition of hydrogen peroxide. The formation of this HOBr in seawater is probably part of a defence mechanism of the plant to prevent the growth of bacteria and fungi on its surface. This anti-growth principle used by the plant can be imitated according to the invention in anti-fouling paints for yachts and ships in both fresh and salt water. Seawater in particular contains considerable quantities of hydrogen peroxide and 1mM of bromide is normally speaking also present. Since the haloperoxidases according to the invention have a high affinity for bromide in addition to an affinity for chloride, the vanadium chloroperoxidase will oxidize the bromide ions to HOBr. A painted surface which contains the enzyme and is exposed to water, particularly seawater, will continuously release the bactericidal agent HOBr and prevent the growth of (micro-)organisms on the surface of ships and the like.

According to a second aspect of the invention a method for determining the chloride concentration in liquids is provided.

Since particular peroxidases have a high affinity for chloride, they are found to be very useful in a new enzymatic method for determining the halide concentration in aqueous solutions. The method can likewise be used for determining the halide concentration in body fluids such as blood and urine. The method according to the invention is very sensitive and can demonstrate concentrations in the μ-molar range. The method according to the invention is preferably based on the already known monochlorodimedone assay. Monochlorodimedone reacts with the product of the enzymatic oxidation of halide to dichloro- or monobromo-monochlorodimedone in the presence of chloroperoxidase and only to the latter compound with bromoperoxidase. The reaction is monitored by measuring the absorption at 290 nm which decreases after chlorination or bromination of monochlorodimedone.

For the applications according to the invention all haloperoxidases can of course be used which produce (disinfecting) hypohalogenic acids when exposed to halide-containing aqueous solutions. In a particularly advantageous embodiment of the invention however, only non-haemo vanadium haloperoxidases are used.

The seaweed Ascophyllum nodosum was the first species wherein a non-haem vanadium bromoperoxidase was discovered. A large number of other seaweed species thereafter followed which were also found to contain these enzymes. The bromoperoxidase from A. nodosum has been extensively studied and characterized (1, 2). The enzyme catalyzes the formation of hypohalogenic acids from the corresponding halogens. In a first step hydrogen peroxide reacts with the enzyme and thus forms a hydrogen peroxide-enzyme complex. Bromide and a proton then react with the complex to form an enzyme-HOBr complex. This complex decomposes and thus provides the enzyme and HOBr (3).

The known vanadium bromoperoxidases are found to display a high operational stability in aqueous and organic media. They can be stored for over a month in organic solvents, such as acetone, methanol, ethanol, 1-propanol,

without loss of activity (4). The applications or possible applications for this type of enzyme are therefore wide.

The bromoperoxidases have the drawback however that bromide has to be present for their activity in such potential applications. This is not however the case in a good many situations. Bromide will therefore have to be added. In addition, attempts to isolate the genes for these bromoperoxidases from seaweed and to determine their sequence have not been successful up to the present. It is therefore not yet possible to obtain recombinant bromoperoxidases in large quantities for commercially viable applications.

Now however, enzymes corresponding with the known bromoperoxidases have been found which are not dependent on the presence of bromide but of chloride and which can be produced in large quantities using recombinant DNA techniques.

It has for instance been found that a vanadium peroxidase, which is found inter alia in the fungus Curvularia inaequalis, can use chloride in addition to bromide in order to be active. In contrast to bromide, chloride is very widely present in for instance tap water, surface water and the like and does not have to be supplied additionally for the action of the enzyme in different applications. Related vanadium chloroperoxidases have now also been found in different Drechslera species, such as Drechslera biseptata, Drechslera fugax, Drechslera nicotiae, and Drechslera subpapendorfii, or the Embellisia species Embellisia hyacinthi and Embellisia didymospora, as well as Ulocladium chartarum and Ulocladium botrytis.

Haloperoxidases in isolated or recombinant form, which can be obtained from any of the aforementioned filamentous fungi or related species, are therefore preferably used in the applications according to the invention.

The aforementioned fungus species can be obtained at the Centraal Bureau voor Schimmelculturen (CBS: Central Institute for Fungal Cultures) in Baarn, The Netherlands via the deposit accession numbers below.

FUNGUS	CBS-ACCESSION NUMBER
Curvularia inaequalis	102.42
Drechslera biseptata	371.72
Drechslera subpapendorfii	656.74
Drechslera nicotiae	655.74
Drechslera fugax	509.77
Embellisia hyacinthi	416.71
Embellisia didymospora	766.79
Ulocladium chartarum	200.67
Ulocladium botrytis	452.72

The vanadium haloperoxidases, which can be isolated from the above fungus species are generally chloroperoxidases. Chloroperoxidases are peroxidases which have an affinity for both chloride and bromide and for iodide and can therefore use these three halides as substrate. The terms "chloroperoxidase(s)" and "haloperoxidase(s)" may be used interchangeably in this application. They have as common characteristic however that they have at least a useful affinity for chloride.

The vanadium chloroperoxidases are found to display a very high thermostability and a high affinity for the substrate. The Tm for the haloperoxidase of Curvularia inaequalis is for instance 90°C. The enzyme is moreover very stable in 40% methanol, ethanol or propanol (7). Its pH-optimum is pH 5.5.

The gene that codes for the haloperoxidase of Curvularia inaequalis has been isolated and its complete sequence determined. Example 3 indicates on the basis of Curvularia inaequalis how the haloperoxidases can be isolated. The genes and sequences of the other haloperoxidases which can be used in the applications according to the invention can be isolated and determined in analogous manner. The sequence

of the chloroperoxidase of Curvularia inaequalis is shown in figure 6.

The purified enzymes of Curvularia inaequalis and Drechslera biseptata were cleaved into peptides by means of proteases and CNBr. The amino acid sequence of two corresponding peptides of both fungi was determined. There was found to be a very high degree of homology between the two species. Of the 21 amino acids, only one was found to differ. (Asp. in C. inaequalis and Glu in D. biseptata at position 14 of the peptide).

In analogous manner the genes and sequences of vanadium haloperoxidases from related fungi can be isolated, determined and expressed.

Starting from the gene sequence, either derived from a cDNA or originating from a genome clone, it is possible to produce a recombinant haloperoxidase by including the sequence in a suitable expression cassette with suitable transcription, initiation and termination signals in addition to a replication origin, in a suitable host such as Aspergillus sp., Saccharomyces spec. or Streptomyces, Bacillus, E.coli. This recombinant enzyme, as well as biologically active derivatives thereof, can also be used in the applications according to this invention.

The present invention will be further elucidated on the basis of the accompanying examples, which are however only given here by way of illustration and are not intended to limit the invention in any way. Examples 1 to 3 show the manner in which suitable haloperoxidases in isolated or recombinant form can be obtained. Example 4 shows that the haloperoxidases actually do have a bactericidal action. Example 5 further explains the paint applications of the invention, while example 6 relates to the new enzymatic chloride assay.

EXAMPLE 1

Demonstration of extracellular chloroperoxidases in a number of fungus species.

1. Material and method

Different fungi obtained from the Centraal Bureau voor Schimmelculturen in Baarn were cultured on agar plates. After the growth had stopped the extracellular proteins were transferred to a nitrocellulose filter by means of blotting. The filters were tested for haloperoxidase activity in an assay with ortho-dianisidine and hydrogen peroxide at different pH values and in the presence and absence of potassium bromide.

2. Results and discussion

Of the tested fungi Curvularia inaequalis, Drechslera biseptata, Drechslera fugax, Drechslera nicotiae, Drechslera subpapendorfii, Embellisia hyacinthi, Embellisia didymospora, Ulocladium chartarum and Ulocladium botrytis were found to display haloperoxidase activity. Chloroperoxidases were isolated from Drechslera subpapendorfii, Embellisia didymospora and Ulocladium chartarum. The pH optima of the enzymes varied from pH 4.5 to pH 5.5. After addition of vanadate the enzymatic activity increases. A polyclonal antiserum that was elicited against the chloroperoxidase of Curvularia inaequalis displayed cross reactivity with the enzymes from Drechslera subpapendorfii, Embellisia didymospora and Ulocladium chartarum.

EXAMPLE 2

Isolation of vanadium chloroperoxidase from Drechslera biseptata.

1. Introduction

A large number of halogenated compounds occur in nature. They are produced by different organisms, such as marine algae, actinomycetes, lichens, fungi, bacteria and higher plants. Bromoperoxidase and chloroperoxidase are for instance involved in the production of such halogenated compounds (6). There are two groups of haloperoxidases, each of them having a different prosthetic group. The one group

contains haem as prosthetic group, for instance the chloroperoxidase from the fungus Caldariomyces fumago. This haem protein is however not stable and its pH-optimum in the chlorination reaction lies at a low pH (8). The other group contains vanadium as prosthetic group. One such peroxidase is for instance secreted by the fungus Curvularia inaequalis (6). Described in this example is the isolation of a vanadium chloroperoxidase from the fungus Drechslera biseptata, which has an unusually high stability.

2. Material and method

2.1. The culture of the fungus.

The fungus D. biseptata, strain number 371.72, was obtained from the Centraal Bureau voor Schimmelculturen (CBS, Baarn, The Netherlands). The germination medium consisted of 15 g fructose, 3 g yeast extract (GIBCO BRL) and 1 ml micro-element solution. (0.8 g KH_2PO_4, 0.64 g $CuSO_4.5H_2O$, 0.11 g $FeSO_4.7H_2O$, 0.8 g $MnCl_2.4H_2O$, 0.15 g $ZnSO_4.7H_2O$ in 1 litre water. The fermentation medium consisted of 5 g casein hydrolysate (GIBCO BRL), 3 g yeast extract and 1 g fructose in 1 litre Millipore water. The fungi were cultured in two stages. Firstly, 50 ml sterile germination medium was inoculated with a trace mass of the fungus. This culture was shaken for three days at 23°C and then transferred to a 3 litre Erlenmeyer flask containing 1 litre fermentation medium. The medium which was shaken at 23°C was collected after 14 to 17 days. The fungus D. biseptata secreted a haloperoxidase into the medium.

2.2. Activity determination

To determine the activity of the secreted peroxidase a qualitative assay was used containing 0.1 M potassium phosphate (pH 6.5), 0.1 M KBr, 40 µM phenol red and 1 mM H_2O_2. Conversion of the orange colour to a deep purple colour, which corresponds with the formation of bromophenol blue (4), is observed in the presence of an active haloperoxidase. The growth medium was subsequently filtered and DEAE-Sephacel was added to bind the proteins present.

The column was washed with 0.2 M NaCl in 0.05 M Tris-SO_4 (pH 8.3) and the enzyme was eluated with 0.6 M NaCl in 0.05 M Tris-SO_4 (pH 8.3). It was noted that fractions with activity contained a dark brown colorant which interfered with the quantitative assay of the chlorination activity and the protein assay. The ionic strength of the concentrated sample of the DEAE-Sephacel column was set at 2 M NaCl in 0.05 M Tris-SO_4 (pH 8.3) and the sample was placed on a hydrophobic interaction column Sepharose CL-4B (Pharmacia LKB Sweden). The brown colorant was removed by washing the column with loading buffer and the enzyme was eluated with a gradient of 2 M to 0 M NaCl in 0.05 M Tris-SO_4 (pH 8.3). The enzyme eluated at about 1.2 M NaCl.

The enzymatic activity of chloroperoxidase in the oxidation of Cl^- to HOCl was determined at 25°C (8) by monitoring the conversion of 50 µM monochlorodimedone (ϵ_{290nm} = 20.2 $mM^{-1}.cm^{-1}$) to dichlorodimedone (ϵ_{290nm} = 0.2 $mM^{-1}.cm^{-1}$) in 0.1 M citrate buffers of different pH and 50 µM monochlorodimedone (Sigma). 1 unit of chloroperoxidase is defined as 1µmol monochlorodimedone chlorinated per minute in a medium with 1mM H_2O_2, 0.1 M citrate (pH 5.0), 50 µM monochlorodimedone and 5 mM potassium chloride. H_2O_2 was prepared by dilution of a 30% stock solution of Perhydrol (Merck, Darmstadt, Germany). The reaction was started by adding the enzyme to the reaction medium.

2.3. Other assays

The method of Bradford (Anal. Biochem. 72, 248-254 (1976)) with bovine serum albumin as standard was used for the protein assay.

SDS polyacrylamide gel electrophoresis was performed with 10% gels as described by Laemmli (Nature 227, 680-685 (1970)). Standard proteins (low molecular weight 14.4-94 kDa, Pharmacia, LKB, Sweden) were used for the molecular weight determination.

The protein staining was performed with Coomassie Brilliant Blue G250.

The bromoperoxidase activity in SDS-PAGE gels was determined by immersing the gels in a solution of 0.1 M potassium phosphate (pH 6.5), 0.1 M potassium bromide, 1mM orthodianisidine and 1 mM H_2O_2. When the peroxidase is present, a brown precipitate is formed in the gel.

The optical measurements were performed on a Cary 17 spectrophotometer.

EPR-spectra (EPR = Electron Paramagnetic Resonance) were recorded on a Bruker ECS-106 spectrometer. The instrument was used at a X-band frequency with 100 kHz magnetic field modulation. EPR-samples were prepared by reduction with sodium dithionite, whereafter the solutions were frozen in liquid nitrogen.

Vanadium was determined with the standard addition method using a Hitachi 180-80 Zeeman polarized spectrophotometer fitted with a Hitachi pyrolysis graphite cuvette. Free and aspecifically bound vanadium were removed by centrifugation of the samples through a column of the cation exchanger Chelex-100 (Biorad) before the quantity of incorporated vanadium was determined.

All chemicals were of analytical quality. The water was filtered and de-ionized by carrying it through an Elgastadt B124 (Elga group) and a Milli-Q (Millipore Corporation) water purification system.

3. Figures

Figure 1 shows the total number of units of chloroperoxidase isolated from media containing different concentrations of vanadate. The activity was determined as described under Material and method.

Figure 2 shows at a number of points in time the chlorination activity of apo-chloroperoxidase which has been reactivated at a respectively low and high ionic strength by vanadate. 75 nanomolar apo-chloroperoxidase was incubated with 100 μM sodium vanadate. □--□ = 0.05 M Tris-SO_4 (pH 8.3) and 0.2 M Na_2SO_4; ◊--◊ = 0.05 M Tris-SO_4 (pH 8.3).

Figure 3 illustrates the thermostability of chloroperoxidase. 0.2 mg/ml enzyme was incubated for 5 minutes at

different temperatures and the chlorination activity was determined.

Figure 4 illustrates the stability of the enzyme in organic solvents. The chloroperoxidase was stored in organic solvents and samples thereof were taken in order to determine the chlorination activity.

Finally, figure 5 shows the EPR-spectra of chloroperoxidase from D. biseptata (curve a, 2.5 mg/ml) and C. inaequalis (curve b, 3 mg/ml) in 50 mM Tris-SO_4 (pH 8.2) after reduction with sodium dithionite. The equipment was adjusted as follows: microwave power 40dB; microwave frequency 9.425 GHz; modulation frequency 0.8 mT, temperature 50 K.

4. Results

The yield of the enzyme was about 10 mg enzyme per litre of fermentation medium. SDS-PAGE under denaturing conditions (boiling in the presence of ß-mercaptoethanol) of the chloroperoxidase preparation showed one important band at 66 kDa (not shown). When the chloroperoxidase sample was not boiled in the buffer with ß-mercaptoethanol, 1 band of 66 kDa and another band with a higher molecular weight were present which both stained for activity and protein. The preparation was therefore pure but the enzyme apparently formed aggregates. A corresponding band pattern was also found for the chloroperoxidase from C. inaequalis.

The chloroperoxidase from C. inaequalis contains vanadium as prosthetic group and when no extra vanadium is added to the growth medium the enzyme is secreted in its apo-form.

The amount of activity in the different fermented materials from D. biseptata fluctuated considerably and this could be attributed to the secretion of the enzyme in its apo-form. In order to test this, different concentrations of sodium orthovanadate were added to a number of culture media at the start of the growth. After 17 days of fermentation the dry weight of the fungus was determined and the chloroperoxidase was isolated from the different culture

media. The samples containing activity were concentrated and the protein content and activity determined. The number of isolated units was a function of the concentration of vanadate present in the medium (see figure 1). A constant activity level was observed when the medium contained more than 10 μM vanadate. Both the dry weight of the fungal material and the quantity of protein of the purified preparation remained the same. Therefore D. biseptata likewise secretes an apo-enzyme when no extra vanadium is added to the medium.

Whether apo-enzyme purified from a medium without sodium orthovanadate could be reactivated was also tested. To this end a sample was incubated with a tenfold excess of sodium orthovanadate and the chlorination activity was measured at different time intervals. Since vanadium bromoperoxidase appears to aggregate easily at low ionic strength, the reactivation was performed in a medium which only contained 0.05 M Tris-SO_4 (pH 8.3) and also in one containing 0.2 M Na_2SO_4 in 0.05 M Tris-SO_4 (pH 8.3). In figure 2 can be seen that sodium orthovanadate activates both samples. At a high salt content the chloroperoxidase is however activated much quicker, which suggests that a low ionic strength causes the formation of aggregates, wherein the reactivation speed is inhibited by sodium orthovanadate.

Since vanadium bromoperoxidase enzymes are relatively stable (4), stability experiments were performed. Figure 3 shows the thermostability of the chloroperoxidase from D. biseptata. From this figure a mean temperature of 82.5°C can be calculated, which indicates that this enzyme has a very high thermostability.

The effect of the chaotropic agent guanidine-HCl was likewise studied. Chloroperoxidase was incubated for 5 minutes in different concentrations of guanidine-HCl, whereafter the chlorination activity was measured. From the data (not shown) a $G_{½}$ of 2.7 M can be calculated, which indicates that the enzyme is not particularly stable in this denaturing agent. Conversely, the resistance to denaturation by organic solvents is considerable. Samples of the

chloroperoxidase from D. biseptata, which were stored in different organic solvents such as methanol, ethanol, acetone and dioxane, remained stable for up to 6 weeks (figure 4).

From a steady state kinetic study of the chlorination activity it was found that for other haloperoxidases a bell shaped pH-optimum was observed (not shown). The position of the pH-optimum is a function of the chloride concentration, as was also observed for the vanadium enzyme from C. inaequalis (6). The pH-optimum shifts from pH 4.5 at 1mM Cl^- to pH 5.5 when the chloride concentration is raised to 100 mM. Table 1 shows the K_m-value for Cl^- and H_2O_2 at different pH values. It is apparent that the affinity for both substrates is high.

Table 1

K_m* for chloride and hydrogen peroxide of the chloroperoxidase of D. biseptata

pH	K_m Cl-(mM0	K_m H_2O_2 (μM)
4.0	0.18	27.2
4.5	0.22	15.2
5.0	0.76	15.4
5.5	0.96	12.2
6.0	1.99	4.11
6.5	15.5	
7.0	19.6	

* The K_m for chloride was obtained by measuring the chlorination speed at saturation levels of hydrogen peroxide and the K_m for hydrogen peroxide at saturating concentrations of chloride. The chlorination activity was determined as stated in Material and method.

The EPR-spectrum of the purified enzyme was also recorded. As is the case for the other vanadium haloperoxidases (1), no EPR-signal is detectable in the oxidized enzyme. After reduction with sodium dithionite a typical vanadyl EPR-spectrum is however observed (figure 5). By way of comparison the EPR-spectrum of the haloperoxidase from C. inaequalis is also shown. The isotropic EPR-parameters g_0 and A_0 are almost the same for both enzymes and correspond with those of the vanadium bromoperoxidases. This data suggests that the prosthetic group in these enzymes is the same.

The demonstration of other vanadium chloroperoxidases in normal soil fungi indicates that vanadium enzymes are widespread in nature.

EXAMPLE 3

Determination of the coding sequence of the CPO gene and the gene from Curvularia inaequalis and expression systems.

1. Material and method

The coding sequence of the CPO gene was determined as follows. The chloroperoxidase from C. inaequalis was cleaved with protease or CNBr to obtain peptides. The amino acid sequence of these peptides was determined using a gas phase sequencer. The resulting sequences are shown in table 2. On the basis of amino acid sequence 1 (see table 2) degenerated primers were designed on both sides of the coding DNA template. Using these two degenerated primers and the genome DNA of C. inaequalis as template, the coding part of amino acid sequence 1 was amplified and cloned in a pUC18 vector. The sequence of this amplified part was subsequently determined. The resulting clone was named pCPO1. The coding sequence of amino acid sequence 2 was obtained in similar manner. The clone resulting therefrom was named pCPO2.

On the basis of these two known sequences the new degenerated primers were designed and used in a PCR experiment with a first strand of cDNA as template. The thus

obtained DNA fragment links the two already known DNA sequences. This total fragment was cloned in a pUC18 vector and the sequence thereof was determined. The resulting fragment codes for parts of the amino acid sequences 1 and 2 and also contains the region that codes for amino acid sequence 3 (see table 2).

In order to obtain the 5'-terminal of the cDNA which encodes chloroperoxidase the 5'RACE kit of Clontech Laboratories (USA) was used. The sequence was determined on the basis of one of the resulting clones (pCPO4). The 3'-terminal of the cDNA was obtained in a PCR making use of cDNA as template. The primers used herein were based on the known DNA sequence and on the NotI-d(T)18 bifunctional primer from a Pharmacia first strand synthesis kit. The resulting 1.4 kb fragment was cloned in pUC18. By means of DNA sequencing it was confirmed that this fragment codes for the C-terminal part of the CPO.

2. Result

Shown in figure 6 is the sequence of the cDNA which codes for the chloroperoxidase from C. inaequalis.

The chloroperoxidase apo-protein can be reactivated again by addition of vanadate (see example 2) and it is therefore probable that no other genes are involved in the incorporation of the prosthetic group in these enzymes.

3. Expression of the chloroperoxidase gene

In order to express the chloroperoxidase the cDNA or a genomic fragment is cloned in known manner in an expression vector. With the resulting expression vector a suitable host cell, such as a fungus, for instance Aspergillus sp., Saccharomyces spec. or bacteria, for instance Streptomyces, Bacillus or E.coli, can be transformed. The culture medium is specifically adapted to the relevant host. The expressed chloroperoxidase can be recovered by known procedures, such as separation of the cells of the medium by centrifugation or filtration, precipitation of protein components in the medium by means of a salt, such as ammonium sulphate,

followed by chromatographic procedures, such as ion exchange chromatography, affinity chromatography and the like.

Table 2

Peptide sequences derived from vanadium chloroperoxidases

Sequence	Cleaving method
C. inaequalis	
1) ML--LYMKPVEQPNPNPGANISDNAYAQLGLVLDRSVLEA[a]	CNBr
2) (S)NADETAEYDDAVRVAIAMGGAQAINS[a]	Trypsin
3) (G)YHPTPGRYKFDDEP	Trypsin
4) IDEPEEYN	Trypsin
5) (D)LRQPYDPTAPIEDQPGIVRT[b]	Trypsin
D. biseptata	
6) LNGLNRDLRQPYDPTAPIEEQPGIV[b]	V8 prot.

[a] underlined sequences were used for designing the degenerated DNA primers.

[b] homologous sequences between C. inaequalis and D. biseptata are printed in bold.

EXAMPLE 4

Assay of the chloride concentration in a liquid.

1. Introduction

Chloride concentrations in natural and waste water are usually determined by means of volumetric methods with silver nitrate or mercury(II) nitrate or spectrophotometrically using mercury(II) thiocyanate and iron(III) ions. Illustrated in this example is the application of a new enzymatic method for determining total halide (with the exception of fluoride) and chloride in aqueous solutions. The method is based on the specific oxidation of halides to hypohalogenic acids, which oxidation is catalyzed by chloro- and bromoperoxidases. The hypohalogenic acid is captured by

monochlorodimedone. The quantitative halogenation of monochlorodimedone is determined spectrophotometrically.

Haloperoxidases form a class of enzymes which are capable of oxidizing halides (X^- = Cl^-, Br^- or I^-) in the presence of hydrogen peroxide to the corresponding hypohalogenic acids in accordance with the reaction:

$$H_2O_2 + X^- + H^+ \rightarrow H_2O + HOX \qquad (1)$$

When a suitable nucleophilic acceptor is present, a reaction will occur with HOX and a halogenated compound will be formed.

Haloperoxidases can be subdivided into chloro-, bromo- and iodoperoxidases in accordance with the most electronegative element that can be oxidized by these enzymes. Chloroperoxidases are thus capable of oxidizing Cl^-, Br^- and I^-, while bromoperoxidases only oxidize Br^- and I^- and iodoperoxidases only iodide.

In this example two vanadium-containing enzymes, namely the chloroperoxidase of the fungus C. inaequalis and the bromoperoxidase from the lichen X. parietina (9) are used to determine total halide (Cl^-, Br^- I^-) concentrations. It will be found from the data that the enzymatic assay of halide concentrations is easy to perform and gives reliable quantitative results.

2. Material and method

The halide content of the aqueous solutions was determined using the monochlorodimedone assay (8). Monochlorodimedone reacts with the product of the enzymatic oxidation of halide to dichloro- or monobromo-monochlorodimedone in the presence of chloroperoxidase and to only the latter compound with bromoperoxidase. The reaction was monitored by measuring the absorption at 290 nm which decreases after chlorination or bromination of monochlorodimedone. At pH 3.6 the extinction coefficient at 290 nm for monochlorodimedone is 15.09 mM^{-1} cm^{-1}, while the extinction coefficients at the same wavelength for di-

chlorodimedone and for monobromo-monochlorodimedone are both 0.1 mM^{-1} cm^{-1}. In the spectrophotometric assay a 50 μM concentration of monochlorodimedone was used. After adding hydrogen peroxide and enzymes to a solution containing less than 50 μM halide a partial absorption decrease was observed. The difference between the initial and final absorption indicates a value for the quantity of halide present.

Addition of chloroperoxidase to the assay will give the total quantity of halide present in the test mixture, while addition of bromoperoxidase gives a value for the halides with the exception of chloride. When the absorption values of the test with chloroperoxidase and that with bromoperoxidase are subtracted from one another, the difference gives the quantity of chloride present in the solution.

All halide assays were performed in 0.1 M citrate buffer (pH 3.6), 1 mM hydrogen peroxide, 50 μM monochlorodimedone and 0.29 μM chloroperoxidase or 0.1 μM bromoperoxidase in a 2.5 ml quartz cuvette using a Cary 17 spectrophotometer. All buffers and solutions were prepared with water which had been filtered and de-ionized by an Elgastadt B12H (Elga group) and a Milli-Q (Millipore) water purification system. All reagents were of analytical grade.

3. Results

Figure 7 shows the decrease in absorption which is observed after conversion of monochlorodimedone by the bromoperoxidase from X. parietina or by the chloroperoxidase from C. inaequalis. The reaction mixture contains 0.1 M citrate buffer (pH 3.6), 1 mM hydrogen peroxide, 25 μM chloride and 15 μM bromide (final concentration of halides: 40 μM). Graph A shows the absorption decrease in the presence of 0.1 μM bromoperoxidase, while graph B shows the absorption decrease in the presence of 0.3 μM chloroperoxidase.

Figure 8 shows the relation between the absorption change at 290 nm and the chloride concentration. The

chloride concentration varies between 4 μM and 32 μM. The enzyme concentration is 0.29 μM. The values shown are average values of three experiments. The relation between the chloride concentration and the absorption decrease is found to be linear.

In table 3 is shown the result of an experiment in which mixtures of bromide and chloride were analysed for bromide and chloride content using the enzymatic assay according to the invention. A comparison of the initially present concentrations of bromide and chloride with the result of the enzymatic assay shows that the enzymatic method measures both halides separately and accurately and is therefore capable of fully converting the halides which are present into hypohalogenic acid. The method is reliable because the measurements only deviate slightly.

With the enzymatic method according to the invention the chloride content of a number of water samples is measured. The results are shown in table 4. It is apparent that the determined values correspond with the given specifications and that accurate data can be obtained since the deviation in measurements is small (4%).

Table 3

Assay of a mixture of chloride and bromide
by the method of the invention.
The total concentration of halides is 24 μM.

Mol ratio chloride and bromide	Determined value (μM) of the total concentration	Determined ratio of chloride and bromide
0.25	24.2 (3.5)*	0.22 (3.0)*
0.67	23.8 (3.1)*	0.70 (2.8)*
4	24.3 (2.8)*	4.2 (3.2)*

*: Relative standard deviation (%). n=3.

Table 4

Chloride content of a number of water samples
The concentration of chloroperoxidase was 0.29 μM, bromoperoxidase was 0.1 μM.

Sample	Measured chloride content (mM)	Reference values (mM)
Spa mineral water	0.15 (3.7)*	0.16_a
Amsterdam tap water	3.1 (4.0)*	$2.1-4.2_b$
Amsterdam canal water	6.5 (2.5)*	

*: Relative standard deviation (%). n=3.
a: as specified on the bottle
b: the value is dependent upon the source, as specified by the Amsterdam Water Board.

4. Discussion

The results show that the new enzymatic method for the quantitative measurement of total halide (with the exception of fluoride) and chloride is simple and clear. The monochlorodimedone and its brominated and chlorinated derivatives have known extinction coefficients and can therefore be used as internal standard for the halide assay. The calibration curve shows a linear relation between the chloride content and the absorption changes in the concentration range between 1 and 35 μM. The method according to the invention is exceptionally sensitive. The concentration range used in the enzymatic assay as according to this example is ten times lower than in the known method of Sagara et al. (Anal. Chim. Acta 270, 217 (1992)). The method of Fajans (Z. anorg. allgem. Chem 137, 221 (1924) is

only accurate when the solutions contain more than 5 mM chloride. This is about a thousand times more concentrated than the solutions which can be determined by the enzymatic method according to the invention. The described method is also more sensitive than that making use of ion-selective electrodes, wherein chloride concentrations of less than 10 μM cannot be measured (10).

The measured bromide/chloride ratios correspond with the ratio of the actual values (see table 3). The results show that the combination of the two vanadium-containing enzymes used in this test provides reliable results, not only in respect of the halide content but also concerning the nature of the halide. When a iodoperoxidase with a high affinity for iodide is used it is even possible to analyze separately the content of any halide in any given mixture.

EXAMPLE 5

The demonstration of the anti-bacterial action of vanadium chloroperoxidase

1. Introduction

With the object of testing the anti-bacterial action of the chloroperoxidase from Curvularia inaequalis, E. coli bacteria (HB101) were exposed to a combination of chloroperoxidase, hydrogen peroxide and chloride in 100 mM NaAc (pH 5.0).

2. Material and method

The E. coli cells, grown in a culture medium (10 g yeast extract, 16 g trypton and 5 g NaCl per litre demineralized water), were washed with a physiological saline solution followed by a washing step in 0.1 M sodium acetate (pH 5.0). 1 ml of this bacteria suspension was taken and added to incubation media containing 0.1 M sodium acetate (pH 5.0), 0 or 10 mM NaCl and 0.05 μM chloroperoxidase and a concentration of hydrogen peroxide of 0, 0.01 mM, 0.05 mM to 0.10 mM. As a control the bacteria suspension was also

incubated in a sterile medium to which no chloroperoxidase (CPO) was added. After incubation for one hour samples of 50 µl were taken and subsequently diluted 10^2x, 10^4x and 10^6x in a physiological saline solution. 100 µl was taken from each of these dilutions and these were placed on agar plates (2% agar in culture medium). After overnight incubation at 37°C the number of bacteria per plate were counted.

3. Results and discussion

Table 5 shows that an incubation in the presence of only hydrogen peroxide also has a bactericidal action. The effect is however increased by adding the chloroperoxidase and surviving bacteria are no longer found at incubation in 0.05 mM hydrogen peroxide.

As is seen from the experiment in which no chloride is added (table 6), the bactericidal action of chloroperoxidase is here also observed. In view of the high affinity for chloride, the enzyme presumably uses the traces of chloride from the buffer system. The greatly declining number of bacteria in the incubation without chloroperoxidase can be the result of very unfavourable conditions (the absence of chloride) during the incubation.

Table 5

Number of surviving bacteria (cells per plate) after incubation with chloroperoxidase in 10 mM Cl^-

H_2O_2 (mM)	no CPO*	0.05 µM CPO*
0	46 10^4	50 10^4
0.01	9 10^4	1.2 10^4
0.05	6 10^4	0
0.10	4 10^4	0

* CPO = chloroperoxidase

Table 6

Number of surviving bacteria (cells per plate) after incubation with chloroperoxidase in presence of extra chloride in the incubation medium

H_2O_2 (mM)	no CPO*	0.05 μM CPO*
0	2.70×10^4	4×10^4
0.01	0.43×10^4	0
0.05	0.08×10^4	0
0.10	0.08×10^4	0

* CPO = chloroperoxidase

The results of this experiment therefore provide proof of the bactericidal action of the chloroperoxidase.

EXAMPLE 6

1. Introduction

This example demonstrates the formation of HOBr by (recombinant) chloroperoxidase from the fungus Curvularia inaequalis immobilized in a paint or polymer system. Based on the bactericidal action demonstrated in example 4, it is possible to extrapolate to a growth-inhibiting action of the paint.

2. Materials and method

In order to determine the activity of peroxidase immobilized in a paint, a qualitative assay was used based on the bromination of 40 μM phenol red to (tetra)bromophenol blue. Conversion of the red colour to a deep purple colour will be observed if the peroxidase system produces HOBr.

This assay enables the formation of HOBr to be demonstrated in a simple visual manner.

Used in the experiments was a recombinant vanadium chloroperoxidase which had been expressed in a yeast strain. The enzymatic and catalytic properties (inter alia the pH optimum and the specific activity of about 20 Units/mg) of this enzyme are identical to those of the original fungal enzyme in its purest form. The original enzyme can of course also be used for the described experiments.

Three test systems were used as model for the growth-inhibiting effect of the paints.

Test system 1

2 grams acrylic latex (Sikkens) was mixed with 100 µl (2mg/ml) chloroperoxidase and this was applied to a piece of wood of 2x2 cm. The paint then contained 0.1 mg enzyme per gram latex. The piece of wood was subsequently dried in air for several hours. In a subsequent experiment a 10 x larger quantity of enzyme was mixed with the paint to 1 mg enzyme per gram latex and dried for the same length of time. The drying process immobilizes the chloroperoxidase.

The pieces of wood were placed in 2 ml medium and carefully shaken with a table shaker to limit diffusion limitation of substrates and products.

Test system 2

A small quantity (about 2 ml) chlorinated rubber anti-fouling 2000 (AKZO) was mixed as well as possible with 0.5 ml chloroperoxidase (2.1 mg/ml). The enzyme preparation dissolved in buffer mixes very badly with the paint. This paint was also applied to a piece of wood and dried for several hours.

Test system 3

To increase the accessibility of the enzyme, a matrix was also used of 20% polyacrylamide in which the chloroperoxidase, in a final concentration of 0.1 mg enzyme per ml acrylamide, was immobilized by causing it to

copolymerize during the polymerization process. For a description of this procedure see Baily J.E. and Ollis, D.F. in Biochemical Engineering Fundamentals. McGraw-Hill Book Company, second edition, pp. 180-202.

The matrix was prepared by mixing 10 ml acrylamide/ bisacrylamide with 0.46 ml chloroperoxidase (2.1 mg/ml). The pores in the matrix system allow easy access of substrates and draining of the formed products, while the enzyme however remains bound to the matrix and thus cannot diffuse therefrom.

To test the formation of HOBr, pieces of gel with a surface area of about 0.5 by 0.5 cm (about 2 mm thick) were cut out and tested in media of 2 ml which were also shaken.

The pieces of wood obtained according to the three test systems were held in a number of media for some time. Used as media were:

Medium A:

100 mM KBr
0.1 M phosphate buffer (pH 6.5)
40 μM phenol red

Medium B:

natural seawater with 40 μM phenol red

3. Results

When the piece of treated wood according to test system 1 is placed in medium A and 1mM of hydrogen peroxide is subsequently added, a blue coloration of the phenol red occurs within 30 minutes as a result of the bromination reactions by HOBr.

When the piece of wood with the higher enzyme concentration is placed in this same medium, an accelerated blue coloration occurs, as expected.

With test system 2 in the same medium only a very slow reaction is measured and the conversion of the phenol red to

bromophenol blue is only measurable after a few days. The slow reaction is probably caused by the poor mixing of this paint with the chloroperoxidase dissolved in water and a small inclusion of the enzyme in the paint. This observation does however show that the enzyme is apparently resistant to the solvent used in the conventional growth-inhibiting agents. It has already been established that the enzyme retains its activity in organic solvents mixable with water (7).

The piece of painted wood from test system 1 (1 mg enzyme per ml latex) was also tested in more natural conditions and placed in 2 ml medium B, to which 1 mM hydrogen peroxide was also added. Here also bromination of phenol red occurs, although the full conversion takes a number of hours. These experiments show that the enzyme is accessible for its substrates (hydrogen peroxide and bromide) via pores in the latex paint and is capable of continuous formation of HOBr.

The polyacrylamide gels in which the enzyme is immobilized also show that HOBr is formed in seawater after addition of hydrogen peroxide. When the seawater (pH about 7.8) is first brought to pH 6.8 with citric acid, a bromination reaction occurs which is complete in about 15 minutes. This is probably the result of the fact that the pH optimum of the chloroperoxidase in the bromination reaction lies at pH 4-6.

The concentration of hydrogen peroxide does not influence the speed of HOBr formation. A test shows that this speed is not affected if the concentration of hydrogen peroxide is reduced to 200 μM.

In all cases a blue coloration of the gel occurs first which spreads further into the medium. To demonstrate that the bromination reactions are not caused by the enzyme leaking out of the matrix, a piece of gel was incubated in 2 ml 1mM Br^- in 0.1 M citrate buffer (pH 6.5) for 3 x 24 hours. After each incubation of 24 hours the medium was changed. When hydrogen peroxide was added to the piece of gel in fresh medium, a rapid bromination reaction once again

occurred which proceeded as rapidly as initially prior to incubation. This data indicates that the enzyme remains immobilized and remains active for a minimum of three days.

4. Discussion

The above described experiments demonstrate that it is possible to immobilize the vanadium chloroperoxidase and that such an enzyme added to paint or a polymer is also capable of formation of HOBr in seawater. The naturally present Br^- (1 mM) is herein oxidized by the enzyme to HOBr in the presence of hydrogen peroxide. It is possible that the enzyme also uses the Cl^- from the seawater, although the Km of the enzyme for bromide is much smaller than for chloride and this means that the chloroperoxidase will preferentially oxidize bromide in a mixture of the two halides.

As demonstrated in example 4, HOBr and HOCl have a strong biocidal action. It will therefore be possible to apply the enzyme system as growth-inhibiting agent in paints.

The second substrate for the enzyme is hydrogen peroxide. It is known from the literature that seawater contains sufficient hydrogen peroxide (11). The concentration amounts to approximately 1 μM and is a result of biological activity and photochemical reactions in seawater under the influence of sunlight. The enzyme is certainly capable of using this low concentration of hydrogen peroxide as oxidizing agent for bromide. The data of Van Schijndel et al. (7) show that the Km for hydrogen peroxide amounts to about 10 μM at pH 5.0 and at higher pH values such as in seawater this Km will decrease still further. This means that very low concentrations of hydrogen peroxide can also be used by the enzyme. The biological activity of adhering micro-organisms can also result in a number of cases in a locally increased hydrogen peroxide concentration. The hydrogen peroxide will be converted effectively into the biocidal HOBr by the enzyme present on and in the painted surface to be adhered to.

This example demonstrates that it is possible to include a haloperoxidase or a mixture of a number of haloperoxidases in a paint without the enzyme losing its activity.

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CLAIMS

1. Paint comprising the usual paint constituents and solvents in addition to a suitable concentration of one or more haloperoxidases.

2. Paint as claimed in claim 1, **characterized in that** the haloperoxidase can be obtained from a fungus chosen from the group consisting of Curvularia inaequalis, Drechslera biseptata, Drechslera fugax, Drechslera nicotiae, Drechslera subpapendorfii, Embellisia hyacinthi, Embellisia didymospora, Ulocladium chartarum, Ulocladium botrytis.

3. Paint as claimed in claim 1 or 2, **characterized in that** the enzyme is the chloroperoxidase from Curvularia inaequalis.

4. Paint as claimed in claim 1, 2 or 3, **characterized in that** the enzyme is a recombinant enzyme.

5. Paint as claimed in claim 4, **characterized in that** the DNA sequence which codes for the recombinant enzyme comprises at least a part of the sequence according to figure 6 or modified versions thereof.

6. Paint as claimed in any of the foregoing claims, **characterized in that** it is a growth-inhibiting paint.

7. Haloperoxidase in substantially isolated form obtainable from a fungus chosen from the group consisting of Curvularia inaequalis, Drechslera biseptata, Drechslera fugax, Drechslera nicotiae, Drechslera subpapendorfii, Embellisia hyacinthi, Embellisia didymospora, Ulocladium chartarum, Ulocladium botrytis for use in a paint as claimed in any of the claims 1-6.

8. Haloperoxidase for use as preservative in paint.

9. Use of haloperoxidase obtainable from a fungus chosen from the group consisting of Curvularia inaequalis, Drechslera biseptata, Drechslera fugax, Drechslera nicotiae, Drechslera subpapendorfii, Embellisia hyacinthi, Embellisia

didymospora, Ulocladium chartarum, Ulocladium botrytis in paint.

10. Use of haloperoxidase as claimed in claim 9, characterized in that the paint is a growth-inhibiting agent, for instance for ships.

11. Method for determining the halide concentration in a liquid, comprising of adding to the liquid for testing a hydrogen peroxide and one or more haloperoxidases, monitoring the oxidation reaction by means of an indicator system and determining the halide concentration on the basis of the indicator system.

12. Method as claimed in claim 11, characterized in that the indicator system is formed by monochlorodimedone.

13. Haloperoxidase in substantially isolated form obtainable from a fungus chosen from the group consisting of Curvularia inaequalis, Drechslera biseptata, Drechslera fugax, Drechslera nicotiae, Drechslera subpapendorfii, Embellisia hyacinthi, Embellisia didymospora, Ulocladium chartarum, Ulocladium botrytis for use in a method for determining the halide concentration in a sample.

14. Use of haloperoxidases in a method for determining the halide concentration in a sample.

15. Test kit for determining the halide concentration in a sample, comprising at least one haloperoxidase, a source of hydrogen peroxide and an indicator system for monitoring the oxidation reaction.

16. Test kit as claimed in claim 15, characterized in that the source of hydrogen peroxide is pure hydrogen peroxide or a source generating hydrogen peroxide and the indicator system is monochlorodimedone.

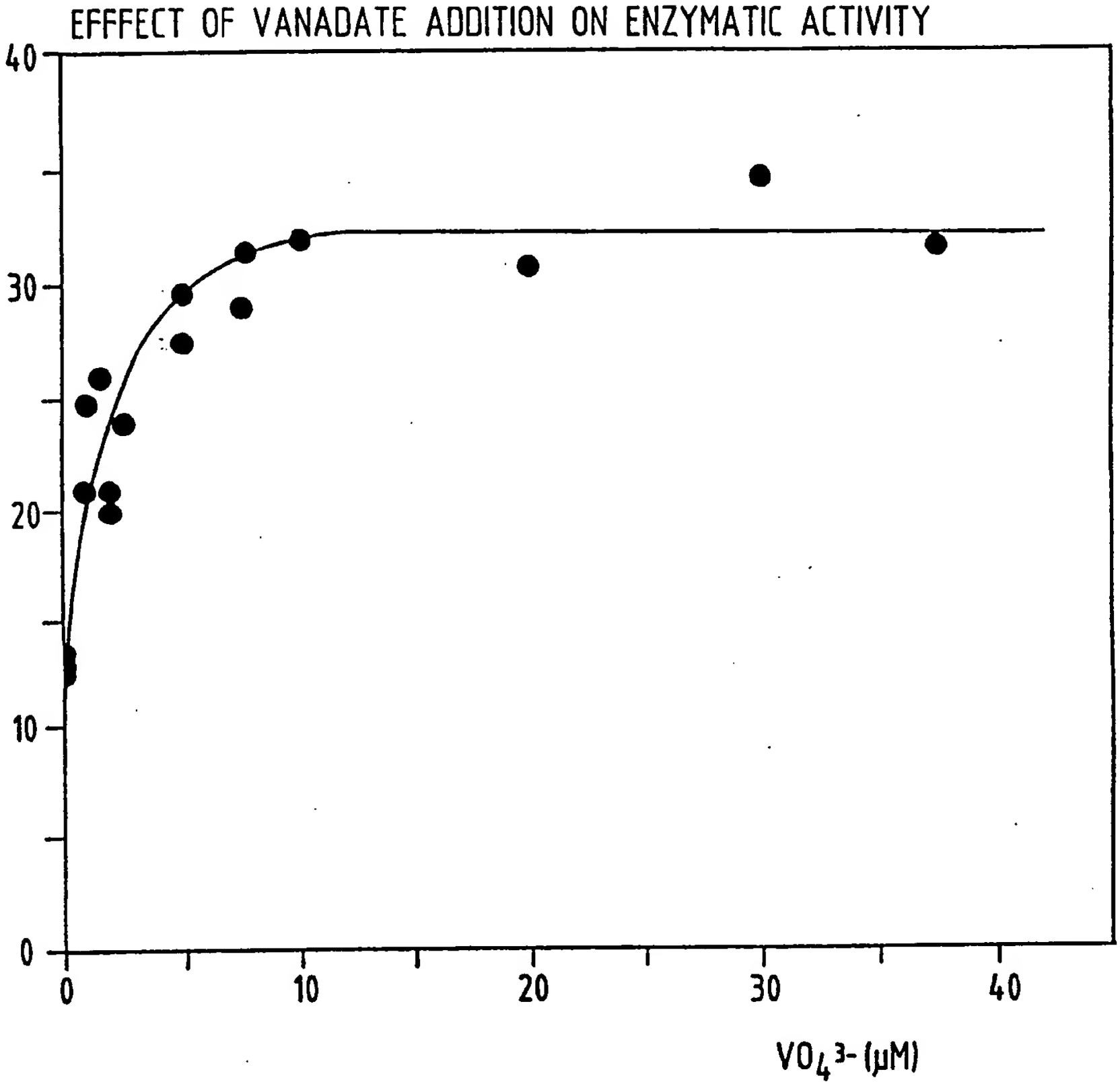
EFFFECT OF VANADATE ADDITION ON ENZYMATIC ACTIVITY
40
30
20
10
0
0
10
20
30
40
VO4 3- (µM)

FIG. 1

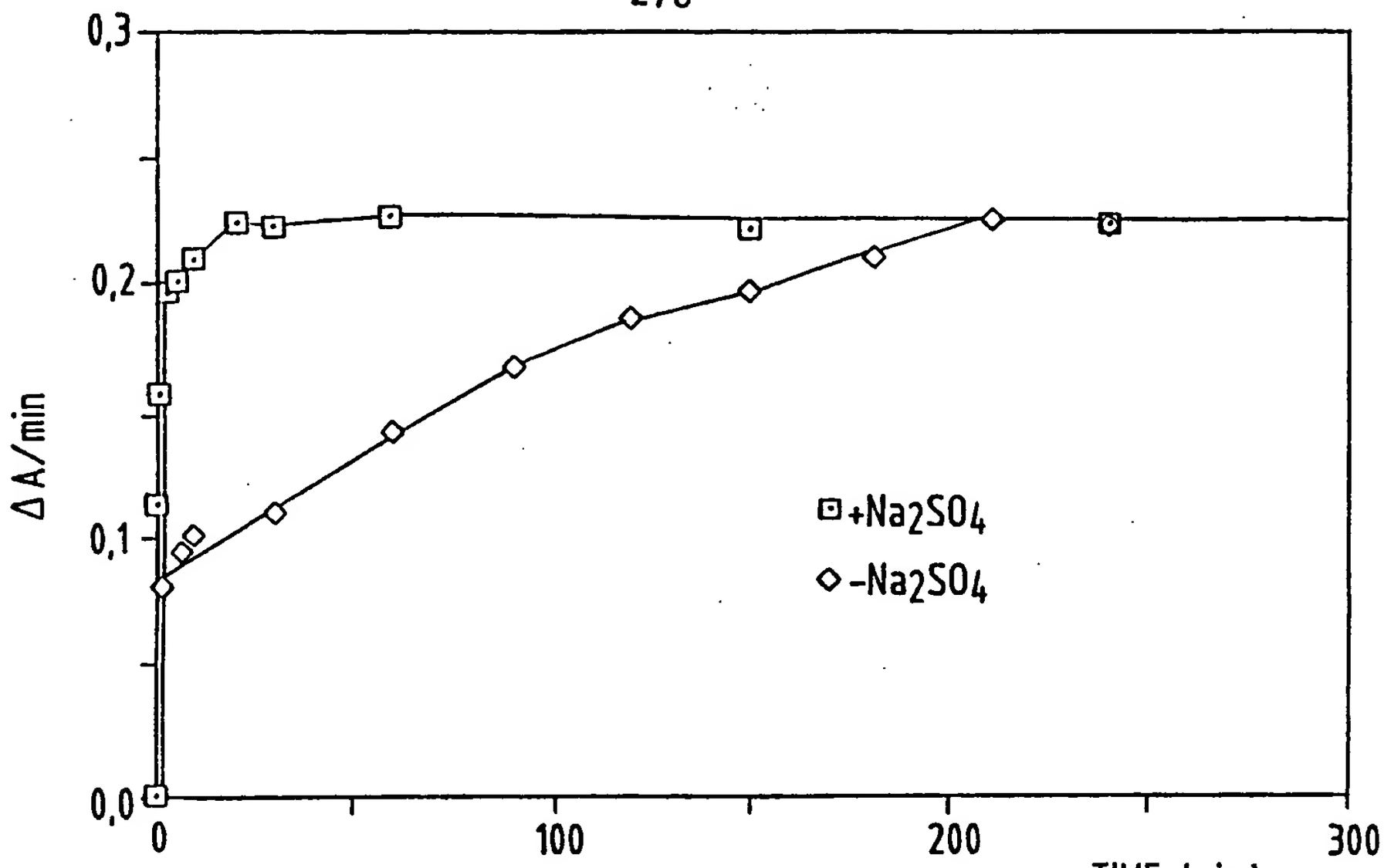
0,3
0,2
0,1
0,0
ΔA/min
0
100
200
300
TIME (min.)
+Na2SO4
-Na2SO4

FIG. 2

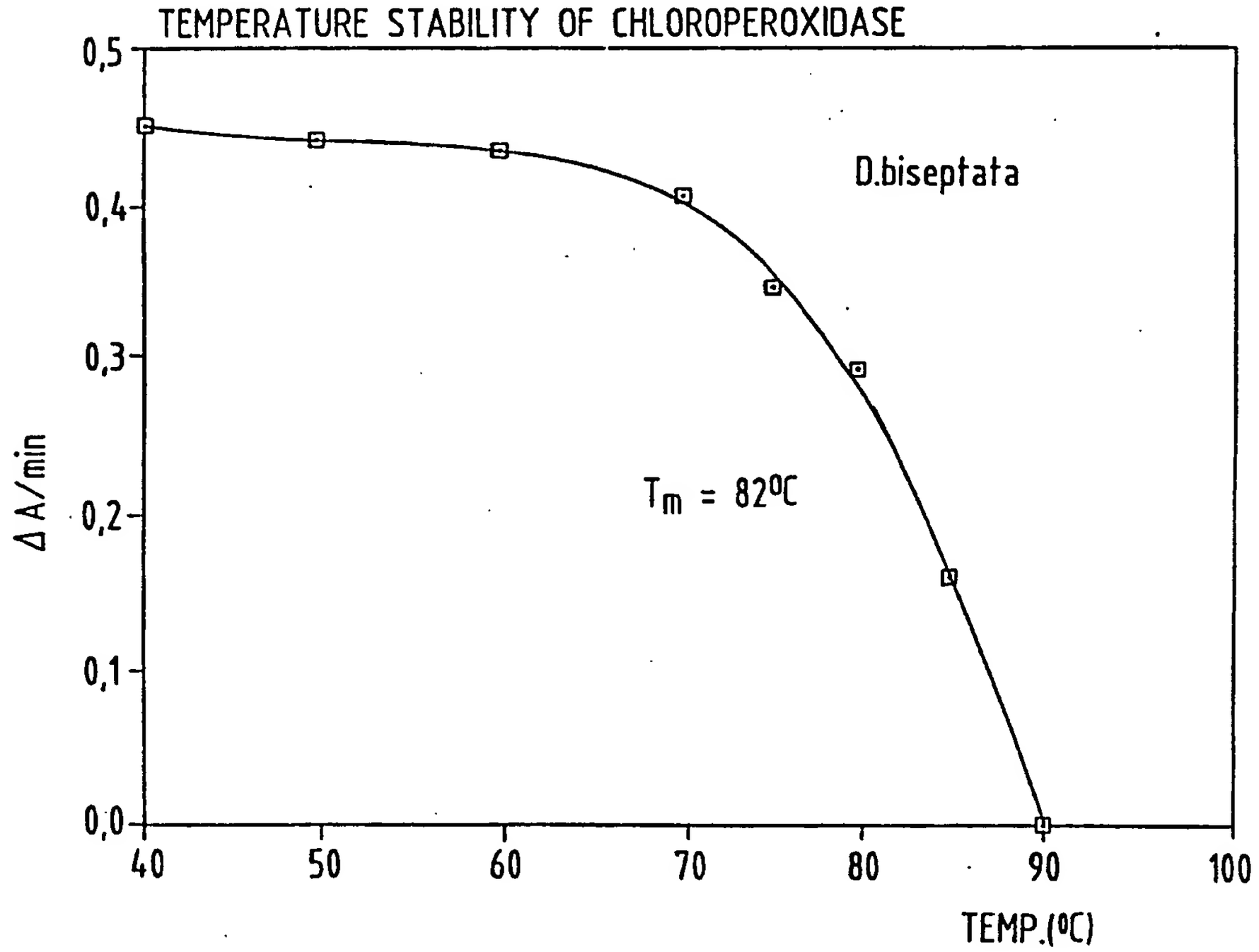
TEMPERATURE STABILITY OF CHLOROPEROXIDASE
0,5
0,4
0,3
0,2
0,1
0,0
ΔA/min
40
50
60
70
80
90
100
TEMP.(°C)
D.biseptata
Tm = 82°C

FIG. 3

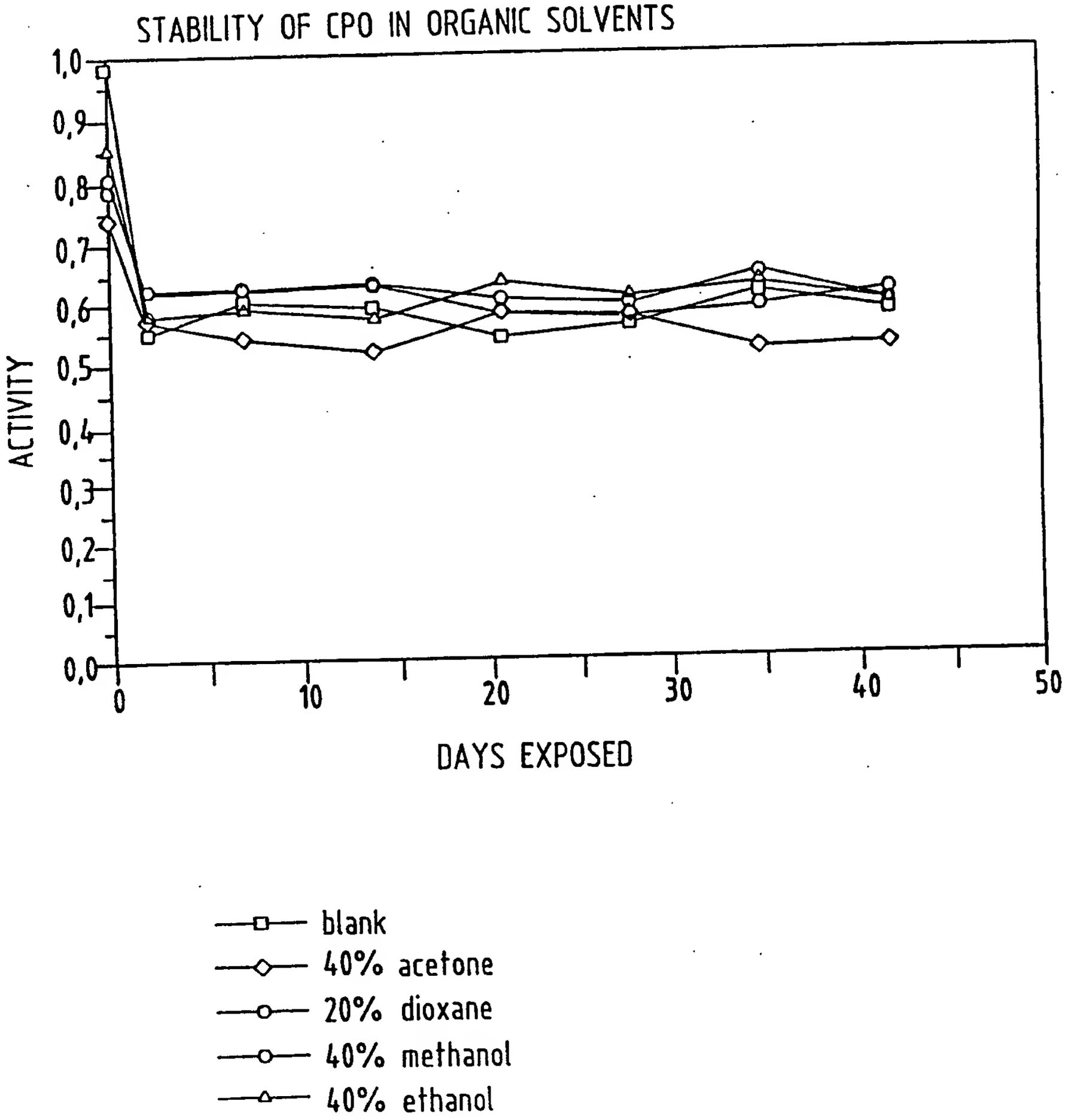
STABILITY OF CPO IN ORGANIC SOLVENTS
ACTIVITY
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
0
10
20
30
40
50
DAYS EXPOSED
blank
40% acetone
20% dioxane
40% methanol
40% ethanol

FIG. 4

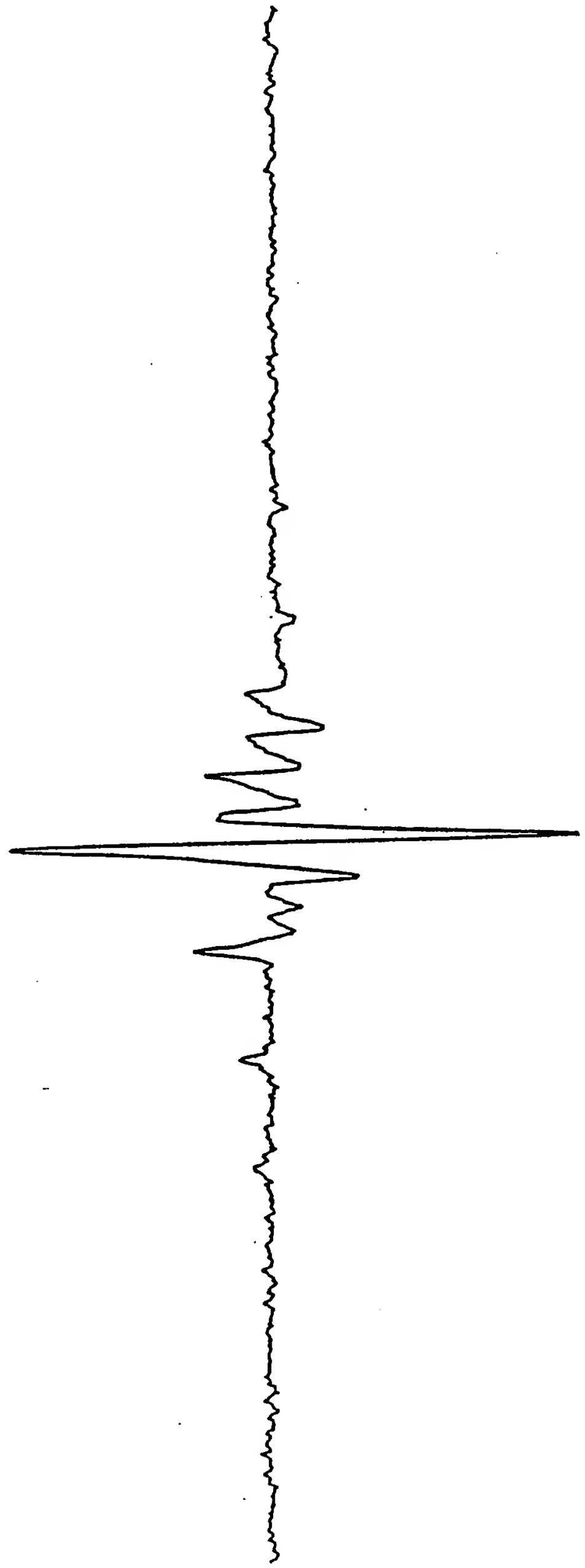

FIG. 5a

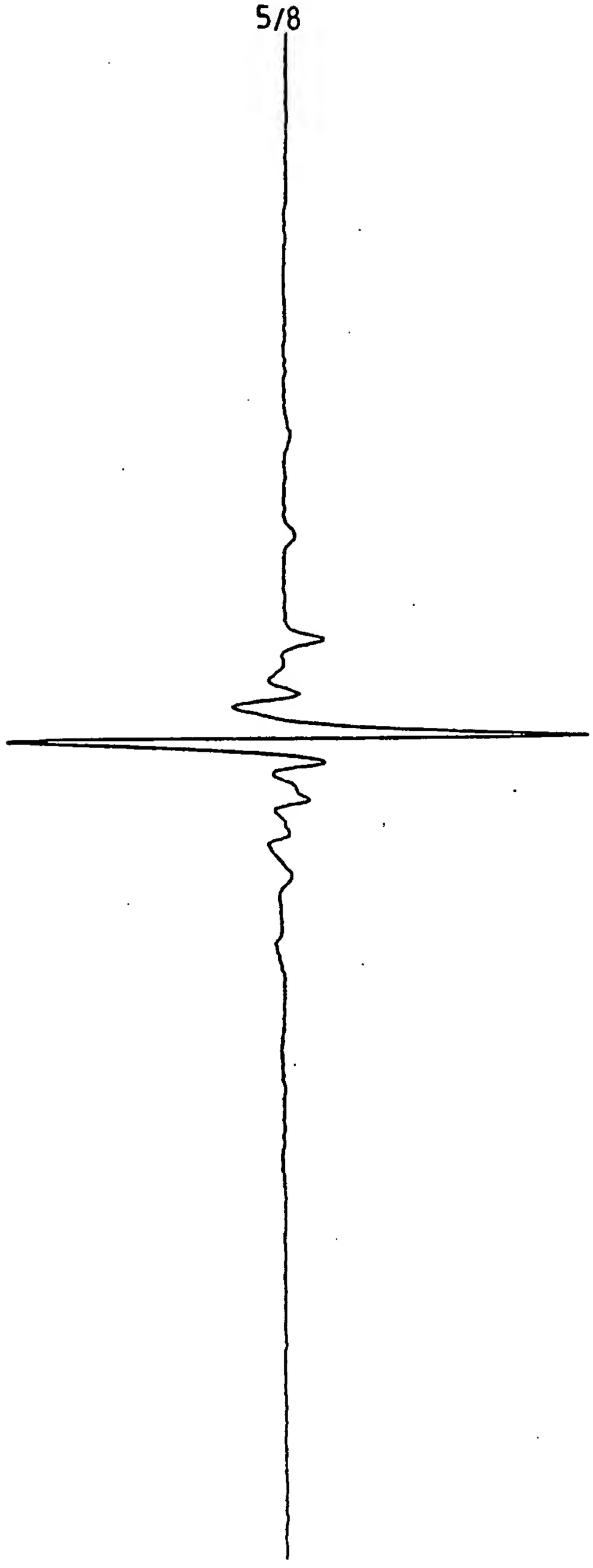

FIG.5b

```
1/1                                                                 31/11
tag tga att gtg gaa tca caa ttc act tgc tca gtc tac tca cta ctt aca atc aca cac
AMB OPA ile val glu ser gln phe thr cys ser val tyr ser leu leu thr ile thr his
61/21                                                               91/31
att atc aca ttc acg atg ggg tcc gtt aca ccc atc cca ctc cct aag atc gat gaa ccc
ile ile thr phe thr     gly ser val thr pro ile pro leu pro lys ile asp glu pro
121/41                                                              151/51
gaa gag tac aac acc aac tac ata cta ttc tgg aac cat gtc ggt ttg gaa ctc aac cgc
glu glu tyr asn thr asn tyr ile leu phe trp asn his val gly leu glu leu asn arg
181/61                                                              211/71
gta act cac act gtt gga ggc ccc ctg acg gga cca cct ctc tct gcc agg gct ctg ggt
val thr his thr val gly gly pro leu thr gly pro pro leu ser ala arg ala leu gly
241/81                                                              271/91
atg ctg cac ttg gct att cac gac gcc tac ttt tct atc tgc cct ccg acc gac ttc acc
met leu his leu ala ile his asp ala tyr phe ser ile cys pro pro thr asp phe thr
301/101                                                             331/111
acc ttc ctc tca cct gat act gag aat gcc gcg tac cgt cta cct agc cct aat ggt gca
thr phe leu ser pro asp thr glu asn ala ala tyr arg leu pro ser pro asn gly ala
361/121                                                             391/131
aat gat gct cgc caa gca gtc gct gga gct gcc ctc aag atg ctg tct tca ctg tac atg
asn asp ala arg gln ala val ala gly ala ala leu lys met leu ser ser leu tyr met
421/141                                                             451/151
aag ccc gtc gag cag cct aac cct aac ccc ggc gcc aac atc tcc gac aac gct tat gct
lys pro val glu gln pro asn pro asn pro gly ala asn ile ser asp asn ala tyr ala
481/161                                                             511/171
cag ctt ggc ttg gtt ctc gac cga tca gtt ctg gag gca cct ggt ggc gtg gac cga gag
gln leu gly leu val leu asp arg ser val leu glu ala pro gly gly val asp arg glu
541/181                                                             571/191
tca gcc agt ttc atg ttt ggt gag gat gta gcc gat gtc ttc ttc gca ctc ctc aac gat
ser ala ser phe met phe gly glu asp val ala asp val phe phe ala leu leu asn asp
601/201                                                             631/211
cct cga ggt gct tcg cag gag ggc tac cac cct aca ccc ggc cgc tat aaa ttt gac gat
pro arg gly ala ser gln glu gly tyr his pro thr pro gly arg tyr lys phe asp asp
661/221                                                             691/231
gaa cct act cac cct gtc gtc ctc att cca gta gac ccc aac aac cct aat ggt ccc aag
glu pro thr his pro val val leu ile pro val asp pro asn asn pro asn gly pro lys
721/241                                                             751/251
atg cct ttc cgt cag tac cac gcc cca ttc tac ggc aag acc acg aag cgt ttt gct acg
met pro phe arg gln tyr his ala pro phe tyr gly lys thr thr lys arg phe ala thr
781/261                                                             811/271
cag agc gag cac ttc ctg gcc gac cca ccg ggc ctg cgt tct aat gcg gac gag acc gcg
gln ser glu his phe leu ala asp pro pro gly leu arg ser asn ala asp glu thr ala
841/281                                                             871/291
gag tat gac gac gcc gtc cgc gtc gct atc gcc atg ggt ggt gct cag gct ctc aac tcc
glu tyr asp asp ala val arg val ala ile ala met gly gly ala gln ala leu asn ser
901/301                                                             931/311
acc aag cgt agc cca tgg cag aca gca cag ggc cta tac tgg gcc tac gat ggg tca aac
thr lys arg ser pro trp gln thr ala gln gly leu tyr trp ala tyr asp gly ser asn
961/321                                                             991/331
ctc att ggc aca cca cct cgc ttt tac aac cag atc gta cgt cgc atc gca gtt acg tac
leu ile gly thr pro pro arg phe tyr asn gln ile val arg arg ile ala val thr tyr
1021/341                                                            1051/351
aag aag gaa gag gac ctt gcc aac agc gaa gtc aac aat gcg gat ttc gcc cgc ctc ttc
lys lys glu glu asp leu ala asn ser glu val asn asn ala asp phe ala arg leu phe
1081/361                                                            1111/371
gcc ctc gtc gac gtc gct tgc aca gac gct ggt atc ttt tcc tgg aag gag aaa tgg gag
ala leu val asp val ala cys thr asp ala gly ile phe ser trp lys glu lys trp glu
1141/381                                                            1171/391
ttc gaa ttc tgg cgc cca cta tct ggt gtg cga gac gac ggc cgt cca gac cat gga gat
phe glu phe trp arg pro leu ser gly val arg asp asp gly arg pro asp his gly asp
1201/401                                                            1231/411
cct ttc tgg ctc act ctc ggt gcc cca gct act aac acc aac gac att cca ttc aag cct
pro phe trp leu thr leu gly ala pro ala thr asn thr asn asp ile pro phe lys pro
1261/421                                                            1291/431
cct ttc cca gtt tac cca tct ggt cac gcg acc ttt ggc ggt gct gtg ttc caa atg gtg
pro phe pro val tyr pro ser gly his ala thr phe gly gly ala val phe gln met val
1321/441                                                            1351/451
cgt cgg ata cta caa cgg ccg cgt agg tac atg gaa gga cga cga acc cga caa cat tgg
arg arg ile leu gln arg pro arg arg tyr met glu gly arg arg thr arg gln his trp
```

FIG. 6

```
1381/461                                                    1411/471
cat cga tat gat gat ctc gga gga gct caa cgg gtg aac cgc gac cta cgc cag tct tat
his arg tyr asp asp leu gly gly ala gln arg val asn arg asp leu arg gln ser tyr
1441/481                                                    1471/491
gac ccc acg gcc cca atc gaa gac caa ccc ggt atc gtg cgc acc cgt att gtt cgg cac
asp pro thr ala pro ile glu asp gln pro gly ile val arg thr arg ile val arg his
1501/501                                                    1531/511
ttc gac tcg ggc tgg gaa ctc atg ttc gaa aac gcc att tcg cgt atc ttc ctc ggt gtc
phe asp ser gly trp glu leu met phe glu asn ala ile ser arg ile phe leu gly val
1561/521                                                    1591/531
cac tgg cgt ttc gat gcc gtc tcc gcc cgc gac att ctc att ccc acg acg aca aag gac
his trp arg phe asp ala val ser ala arg asp ile leu ile pro thr thr thr lys asp
1621/541                                                    1651/551
gtc tac gct gtc gac aac aat gtc gcc ccc gtg ttc cag aac gta gag gac att agg tac
val tyr ala val asp asn asn val ala pro val phe gln asn val glu asp ile arg tyr
1681/561                                                    1711/571
aca ccc agg ggg acg cgt gtg gac ccc gag ggc ctc ttc cct atc ggt ggt gtg cca ctg
thr pro arg gly thr arg val asp pro glu gly leu phe pro ile gly gly val pro leu
1741/581                                                    1771/591
ggt atc gag att gcg gat gag att ttt aat aat gga ctt aag cct acg ccc ccg gag atc
gly ile glu ile ala asp glu ile phe asn asn gly leu lys pro thr pro pro glu ile
1801/601                                                    1831/611
cag cct ata ccg cag gag aca ccg gtg cag aag ccg gtg gga cag cag ccg gtt aag ggc
gln pro ile pro gln glu thr pro val gln lys pro val gly gln gln pro val lys gly
1861/621                                                    1891/631
atg tgg gag gaa gag cag gcg ccg gta gtc aag gag gcg ccg     atg agg tat cgt gag
met trp glu glu glu gln ala pro val val lys glu ala pro     met arg tyr arg glu
1921/641                                                    1951/651
agt atg ggg tag gcg tga gtg gga cgt ctt gga gta agg gct gat gcg gaa gtt tag ttt
ser met gly AMB ala OPA val gly arg leu gly val arg ala asp ala glu val AMB phe
1981/661                                                    2011/671
gat tgg tac ggg tct ggt tta ggg cgt gcg ctc gat act ctg cgg tta ata cac cta ttt
asp trp tyr gly ser gly leu gly arg ala leu asp thr leu arg leu ile his leu phe
2041/681                                                    2071/691
cga tat tag ata gaa tgc tca gat acc tag aat gtg atg att cag ctg tct
arg tyr AMB ile glu cys ser asp thr AMB asn val met ile gln leu ser
```

FIG. 6 (CONTINUED)

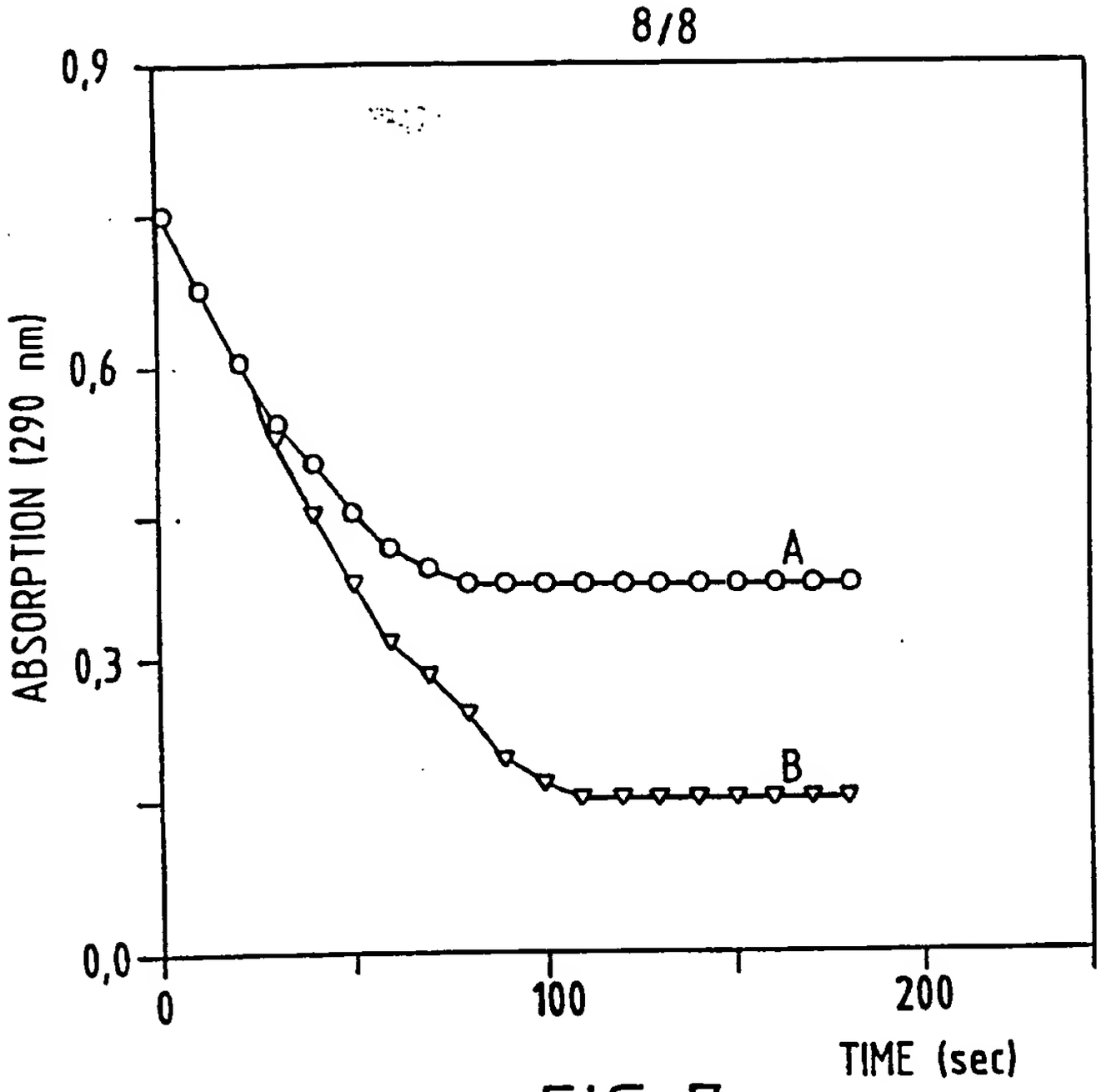
0,9
0,6
0,3
0,0
ABSORPTION (290 nm)
A
B
0
100
200
TIME (sec)

FIG. 7

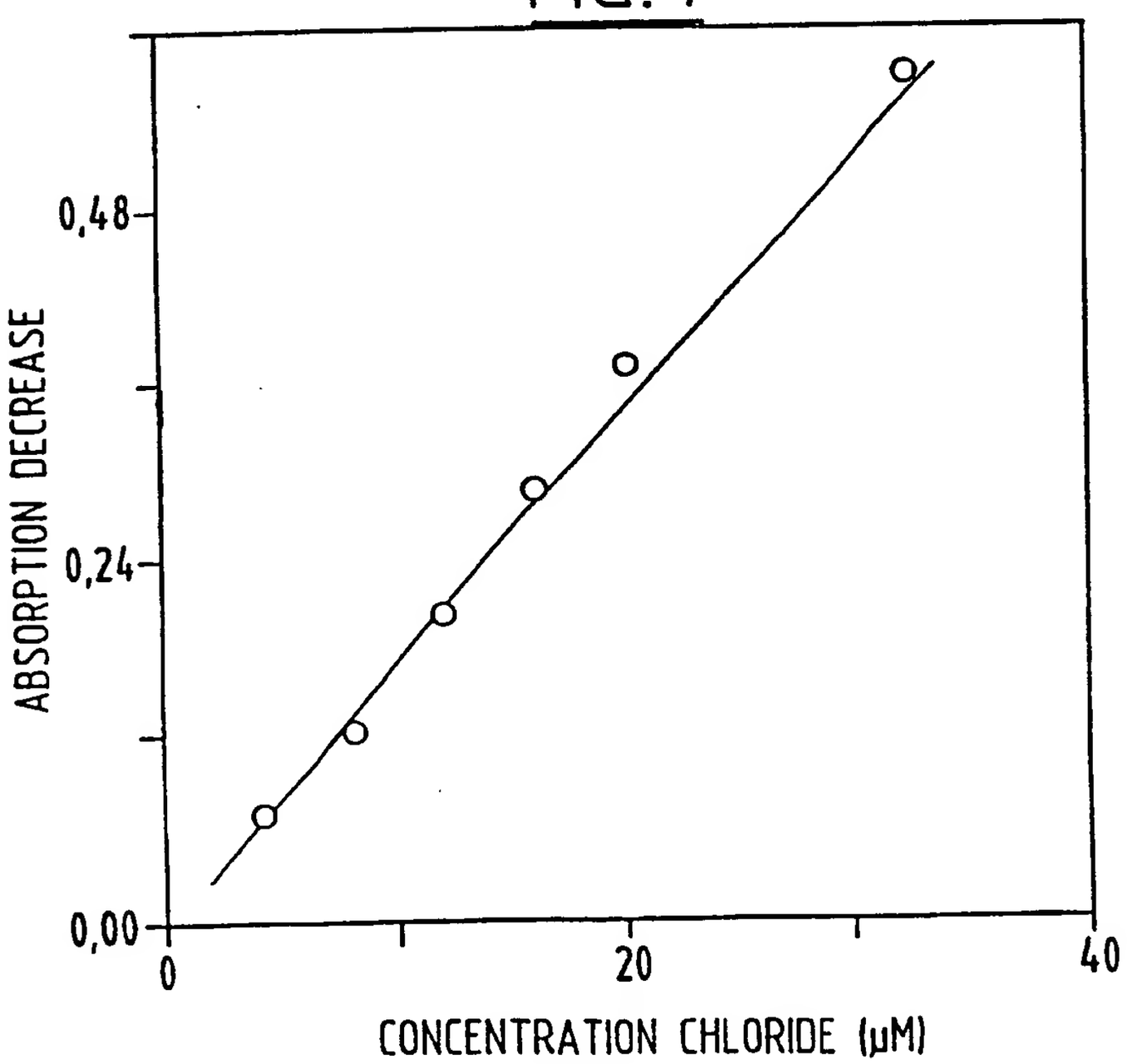
0,48
0,24
0,00
ABSORPTION DECREASE
0
20
40
CONCENTRATION CHLORIDE (μM)

FIG. 8

INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/NL 95/00123

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C09D5/14 C12N9/08 C12Q1/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C09D C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE WPI Section Ch, Week 9406, Derwent Publications Ltd., London, GB; Class A82, AN 94-045582 & JP,A,6 001 933 (MITSUBISHI HEAVY IND CO LTD) 11 January 1994 see abstract ---	1-10
Y	DATABASE WPI Section Ch, Week 8632, Derwent Publications Ltd., London, GB; Class D15, AN 86-209688 & JP,A,61 143 587 (CHIYODA CHEM ENG CO) see abstract --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 June 1995	19. 09. 95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer VAN DER SCHAAL C.A.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/NL 95/00123

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 500 387 (EXOXEMIS) 26 August 1992 see the whole document especially the abstract and page 3, lines 38-48	1-10
Y	DATABASE WPI Section Ch, Week 9242, Derwent Publications Ltd., London, GB; Class A25, AN 92-346279 & JP,A,4 252 284 (IDEMITSU PETROCHEM CO) 8 September 1992 see abstract	1-10
Y	DATABASE WPI Section Ch, Week 8839, Derwent Publications Ltd., London, GB; Class C03, AN 88-275444 & JP,A,63 202 677 (MITSUBISH YUKA BADI) 22 August 1988 see abstract	1-10
Y	FR,A,2 562 554 (NOEL ROLAND) 11 October 1985 see the whole document	1-10
Y	BIOCHIM. BIOPHYS. ACTA, vol.1161, no.2-3, pages 249 - 256 J. VAN SCHIJNDEL ET AL 'The chloroperoxidase from the fungus Curvularia inaequalis; a novel vanadium enzyme' see the whole document	2-7,9,10
Y	US,A,4 707 446 (J. GEIGERT ET AL) 17 November 1987 see the whole document	2-7,9,10

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL95/00123

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 1-10
- claims 11-15

See (1) additional sheet ISA/210

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-10.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

LACK OF UNITY OF INVENTION

1. Claims: 1-10: Use of haloperoxidases in paints.

2. Claims: 11-15: Method to determine the halide concentration plus test kit therefor.

Haloperoxidases has been described in f.i. US4707446 (from Curvularia inaequalis and Drechslera sp.) and Biochim. Biophys. Acta 1161(1993)249-256. Also several applications of these enzymes have been described. See f.i. US4707446 and EP500387.

The problem of the present application is to provide novel applications for haloperoxidases.

Two solutions for this problem have been proposed:

1. Use of haloperoxidases in paint as antifouling agent (Claims 1-10).
2. Use of haloperoxidases in the determination of halide concentrations in liquid (Claims 11-16).

The ISA considers that due to the fact that haloperoxidases already exist and due to the essential difference of the two proposed applications of these enzymes, there is no special technical feature linking together the two uses.

Consequently there is no single inventive concept underlying the different claimed inventions and thus lack of unity within the sense of Art 17(3) (a) PCT.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatic Application No
PCT/NL 95/00123

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0500387	26-08-92	AU-A-	1536492	15-09-92
		CA-A-	2061601	22-08-92
		JP-T-	6505482	23-06-94
		WO-A-	9214484	03-09-92
		US-A-	5389369	14-02-95
FR-A-2562554	11-10-85	NONE		
US-A-4707446	17-11-87	CA-A-	1212061	30-09-86
		US-A-	4937191	26-06-90